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14. ABSTRACT Chemotherapy against Arthropod-transmitted parasitic diseases is increasingly limited by the emergence of drug resistance. Development of new drugs has been slow, emphasizing the need for better, rational anti-parasitic drug discovery. Here we report our discovery of two new potassium ion (K+) channel genes in the genome databases of Plasmodium falciparum. **MAJOR FINDINGS:** To date, we have cloned genes encoding 10 potential K+ channel genes (2-each for *P. falciparum*, *T. gondii,* and 3 each for *L. major* and *T. cruzi*). Using a combination of cultured mammalian cells and *Xenopus* oocytes for heterologous expression we have evidence that 2 channels from malaria [PFK1 &PFK2] and Leishmania [LMK1 & LMK2] generate K+-selective conductances that are sensitive to block by antimalaria drugs chloroquine, quinidine and the K-channel blocker trifluoroperazine. Antisera against PFK1 and PFK2 recognize appropriately sized proteins from *P. Falciparum.* Immunofluorescence of malaria-infected erythrocytes shows that PFK1 islocalized the host cell membrane while PFK2 is primarily associated with the parasite. We also showed that a series of K-channel blockers was capable of killing cultured malria. Our results provide the first report of a cloned ion channel from an intracellular human parasite. Moreover, electrophysiology confirms their identity as K+ channels and pharmacology supports their potential as targets for drug therapy.

15. SUBJECT TERMS
Malaria, Leishmania, potassium channel, chemotherapy

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INTRODUCTION:

The purpose of this research project is to characterize the new gene products that we have identified in *Plasmodium falciparum* as potential potassium channels and to determine if they may be exploited as chemotherapeutic targets. Our planned work included: complete cloning of two *P. falciparum* K^+ channels; subcloning of channel genes for expression and epitope tagging; expression of the channel genes for biophysical, pharmacological and biochemical analyses; generation of specific antibodies to channel proteins for analysis of channels in vivo; and pharmacological analysis of specific K^+ channel blockers as anti-malaria agents. This report describes the progress we have made towards our goals in the first year of funding.

BODY:

Statement of Work

1) *Complete the cloning of PFK1 & PFK2 cDNA to construct expression plasmids for further studies.*

In our search we initially used the universally conserved K^+ channel pore selectivity sequence (T[V/I]GYGD[V/I]) in genome database BLAST screens. We further refined our results by applying the criterion that this selectivity filter must lie in close proximity between 2 predicted hydrophobic helices of 18-28 amino acids in length. With these requirements we have identified (to date) 10 potential K^+ channel gene sequences (2-each for *Plasmodium falciparum* [PFK1 & PFK2], *Toxoplasma gondii* [TGK1 & TGK2], and 3 each for *Leishmania major* [LMK1, LMK2, & LMK3] and *Trypanasoma cruzi* [TCK1, TCK2, & TCK3] **see table**). We have cloned complete cDNAs of each of these channel genes and sub-cloned them into expression and tagging vector plasmids. Due to the unusual codon usage of malaria genes we completely re-synthesized the PFK1 and PFK2 for optimized expression in vertebrae expression systems.

TABLE

Name	Organism	Disease	Source	Genome ID	Protein ID	Protein (AA)	M.W.
Pfk1	*P. falciparum*	Malaria	www.plasmoBD.org	PFL1315w	AAN36349	1935	232kDa
Pfk2	*P. falciparum*	Malaria	www.plasmoBD.org	PF14_0622	AAN37202	1462	174kDa
Lmk1	*L.major*	Leishmanaiasis	www.genedb.org/genedb	LmjF01.0810	AAC24688	1017	114kDa
Lmk2	*L.major*	Leishmanaiasis	www.genedb.org/genedb	LmjF01_0820	AAC24689	1159	127kDa
Lmk3	*L.major*	Leishmanaiasis	www.genedb.org/genedb	LmjF14.0540	LmjF14.0540	499	56kDa
Tgk1	*T. gondii*	Toxoplasmosis	www.toxodb.org	TGG_995368	TgTigrScan2014	1492	165kDa
Tgk2	*T. gondii*	Toxoplasmosis	www.toxodb.org	TGG_995364	TgTigrScan0054	1573	171kDa
Tck1	*T. cruzi*	Chaga's Disease	www.tigr.org/tdb/e2k1	8533.m00006	8533.m00006	1152	131kDa
Tck2	*T. cruzi*	Chaga's Disease	www.tigr.org/tdb/e2k1	8633.m00003	8633.m00003	515	59kDa
Tck3	*T. cruzi*	Chaga's Disease	www.tigr.org/tdb/e2k1	XM_815288	XP_820381	869	99kDa

The predicted amino acid sequences of those structures that comprise the conductivity pathway (selectivity filter and inner helix) all have reasonable homology to known K^+ channels (**Figure 1**). The Leishmania channels (LmK1 and LmK2) contain key glycine residues in the inner helix that are often seen in voltage-gated K^+ channels. Proline residues are present in the inner helices of the Toxoplasma channels (TgK1 and TgK2) and LmK1, LmK2 that may represent bends or angles within the helix as seen in some other K^+ channels. Aromatic residues are present in the malaria in the inner helices of the malaria channels (PfK1 and PfK2), the 3rd T. cruzi channel (TcK3) and LmK1 that are analogous to a site in the HERG channel that is responsible for high-affinity block by organic molecules [*46*]. With the exception of

LmK3, the overall predicted structure of these parasite channels show 6 transmembrane segments as is commonly seen in cationic channels (**Figure 2**). The 4th transmembrane segment of voltage-gated K+ channels generally has charged amino acids (R or K) spaced every third residue for a total of 4-8, which forms the voltage-sensor for gating. The parasite channels variably have between 1 and 4 such amino acids, therefore voltage-gating may not be comparable to channel from higher eukaryotic channels. Based on sequence homology these channels have a modest degree of resemblance to the class of calcium-activated K^+ channels (broadly termed KCa but also known as MaxiK, slo, or BK, IK and SK channels) as well as Shaker-type voltage-gated K^+ channels. KCa channels conduct K^+ ions in response to membrane voltage changes and elevations of intracellular calcium. LmK3 appears to have 2 transmembrane segments flanking the selectivity filter, an arrangement resembling inward rectifier-type K^+ channels.

```
               Selectivity
                 Filter          Inner Transmembrane Helix
Kv1.4   VVTMTTVGYGDMKPITVGGKIVGSLCAIAGVLTIALPVPVIVSNFNYFY
Kir2.1  IETQTTIGYGFRCVTDECPIAVFMVVFQSIVGCIIDAFIIGAVMAKMAK
HERG    FSSLTSVGFGNVSPNTNSEKIFSICVMLIGSLMYASIFGNVSAIIQRLY
huBK    MVTMSTVGYGDVYAKTTLGRLFMVFFILGGLAMFASYVPEIIELIGNRK
PfK1    VITMSTVGYGDYTPVTKAGKFLTMFIIITCISFVAAQFKRLKEAMFSPK
PfK2    IISISTVGYGDIFPINKLSKVVCIIFIFWTFIWVPIQFNDLIISIFSKK
LmK1    IVAFATIGFGDVTAFTTPARIFMIAFIVIGICFFLPLFQRLSVIAERSQ
LmK2    LLSFSSIGYGDVTPLTVQGRLLMIIFIGVGFSYFVPILQYVADLGVYHL
LmK3    FQSLSTLGYGDITPSTLSGRAVAFIAWIVSYVIMAFLIITMYSLLQASD
TgK1    IVTVMTVGYGDFVPRSAAGRALAIITIISTFAFLPGEVQRLMEALREPR
TgK2    VVTMSTVGYGDISPRTMTGQCFCIAFIVTALIWLPSEFGRLIESLTSRR
TcK1    VVTISTVGYGDIIPHGLDGHFLAIVIICVFLAMMSSLIMLVISTTQILR
TcK2    FQAISTVGYGDITPATTAGKLVAFIAWVISFVLIAYLVVIFHGSLTKDE
TcK3    VVTFSTIGYGDIVPGSTPSRLLMAVFIVFAVSHFMPLFQRLVSISRQRL
```

Figure 1. Amino acid sequence alignment of channel conduction pore structures. **G** = suggested flexibility point for voltage-gated K+ channels. **P** = possible bend-inducing prolines. **F** = sites for possible drug block. Kv1.4 = Shaker-type K^+ channel, Kir2.1 = inward rectifying channel, HERG = delayed rectifier channel (human ether a-gogo-related gene), huBK = large conductance calcium-activated K channel.

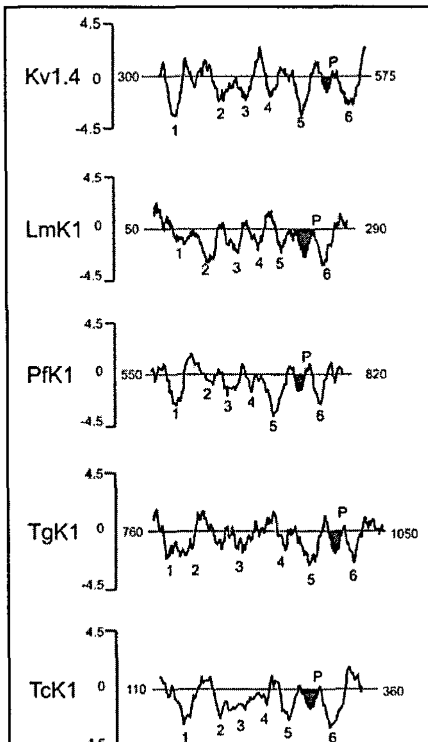

Figure 2. Hydropathy plots of cloned parasite K+ channels. Just the predicted transmembrane segments in proximity to the predicted pore regions are shown. Each channel shows 6 probable membrane-spanning segments with the conduction pore between the 5th and 6th segment. This overall arrangement is conserved among many cation channel types as shown by Kv1.4 on the top panel. Graphs show amino acid analysis with hydrophobic segments below the horizontal lines. The selectivity filter is filled in green.

2) *Express each of the malaria K^+ channel clones in heterologous cell systems for functional assay (electrophysiology) and biochemical detection (immunoblot of epitope tagged recombinant proteins).*
3) *Biophysical (electrophysiology) characterization of the ionic currents generated by each of the malaria K^+ channel clones expressed in a heterologous system.*

Proteins from parasitic protozoa are notoriously difficult to express in heterologous systems. This may be due to unusual codon usage or amino acid sequences not normally seen in cells of the expression systems resulting in either protein degradation, or cell toxicity. We now have successfully recorded K^+ conductance from *Xenopus laevis* (frog) oocytes injected with *in vitro*-transcribed cRNA of LmK1 (**Figure 3**). These currents appear to increase with elevations of cytosolic calcium (well above the endogenous calcium-activated chloride current of oocytes) and the exibit time- and voltage-dependent activation and inactivation kinetics. Due to initial difficulties expressing the PfK genes we completely re-synthesized the cDNAs to optimize codon usage of vertebrate systems. We are now obtaining currents from CHO cells transfected with plasmids containing PfK1 (**Figure 4**). These currents have reversal potentials near the equilibrium potential of K^+ and exhibit an unusual rectification limiting conductance at positive potentials. We are now in the process of fully characterizing the biophysics and beginning pharmacological studies on these currents.

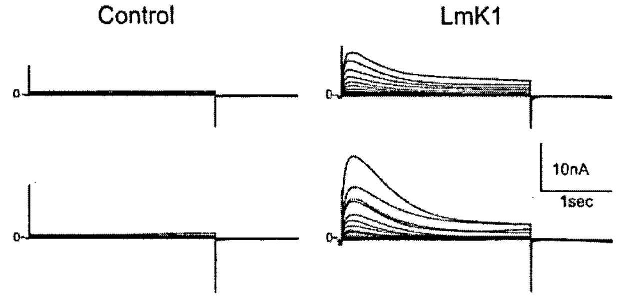

Figure 3. Functional expression of LK1 channels in *Xenopus* oocytes. Oocytes were injected with either aqueous vehicle (left traces) or cRNA from LK1 gene (right traces). Currents are in response to a series of voltage steps between -80 and 80mV. 0 = zero current level.

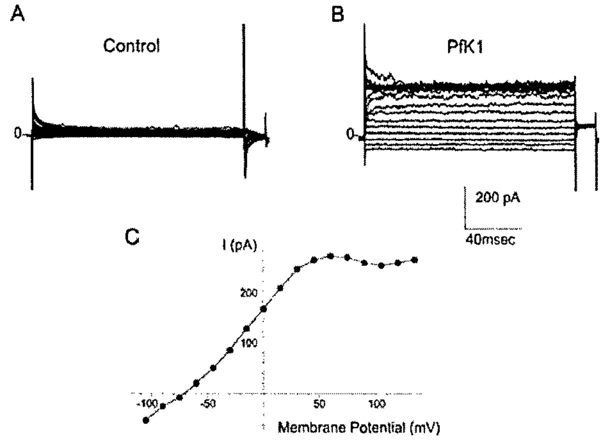

Figure 4. Functional expression of PFK1 channels in CHO cells. Cells were transfected with PfK1 containing plasmid (plus 1/10th the amount of GFP plasmid for identification) by Lipofectamine 2000 (Invitrogen). 48 hours after transfection cells were examined by whole-cell patch clamp analysis. Families of currents are shown in response to voltage-clamp steps from -110mV to +120mV. **A,** currents from control GFP-transfected cell. **B,** currents from PfK1 transfected cell. **C,** current-voltage relationship from PfK1 cell.

4) *Pharmacological screening of drugs that block the cloned K^+ channel currents as measured in a heterologous expression system.*

5) *Biological testing of drugs identified above for anti-parasite activity in cultured Malaria (Plasmodium falciparum. Drugs will be assayed for effects on parasite growth, viability, ability to invade host cells, and ability to successfully exit host cells.*

Just as we have made progress in the functional expression of the channel proteins we have also begun to test a variety of compounds for their ability to block currents from the channels. So far, we have determined that chloroquine completely blocks PFK1 and PFK2 at concentrations as low as 750nM. Quinidine as well, completely blocked the malaria channel currents at concentrations as low as 1uM. We examined a drug, trifluoroperazine (TFP), a psychiatric medication that is known to have activity against calcium-activated K^+ channels from other systems. TFP completely blocked PFK1, PFK2, and LK1 at concentrations as low as 500nM. These are encouraging results that encourage us to pursue both standard anti parasite medications, and known K^+ channel blocking drugs. Moreover, with better constructed cDNAs we are making plans to develop a higher through-put screen of new compounds for activity against the channels.

Given the sequence similarities of the PfK channels to calcium activated K^+ channels we have administered a variety of KCa-blocking drugs in cultures of *P.falciparum* to test for anti-parasite activity. Several agents exhibited anti-malarial activity (in ^3H-hypoxanthine uptake and Giemsa-stain assays). The active drugs included quinidine, clotrimazole, tubocurare, trifluoroperazine, 4-aminopyradine, and haloperidol. The compounds that lacked anti-malarial activity included apamine, charybdotoxin, and iberiotoxin (**Figure 5**). The IC_{50}s for parasite killing for the effective drugs were all comparable to the known IC_{50}s for K^+ channel block. Taken together, these data support the possibility that calcium-activated K^+ channel(s) may be a pharmacological target for anti-malarial chemotherapeutics.

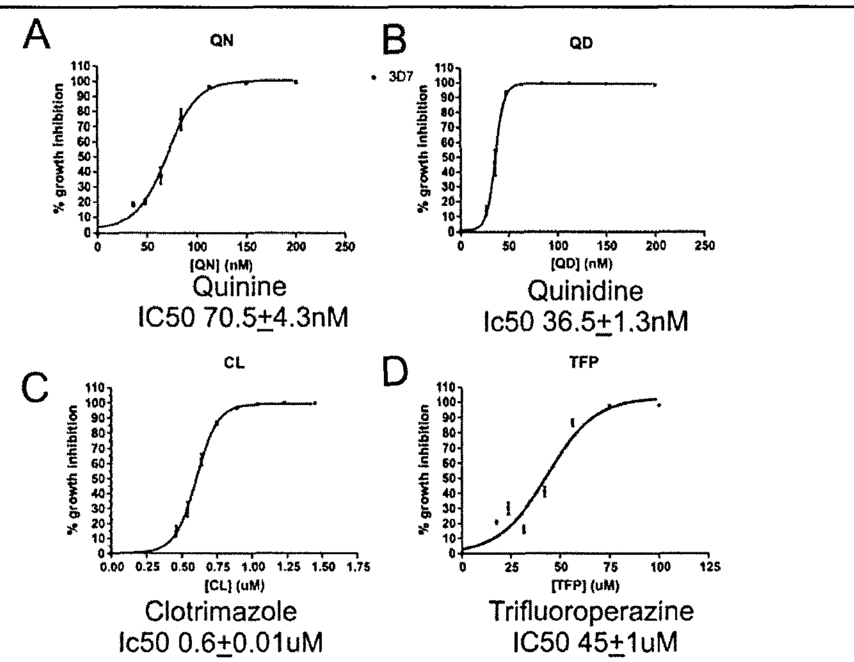

Figure 5. Dose-response curves from *P.falciparum*-infected human erythrocytes. Parasite growth assayed by ^3H-hypoxanthine incorporation over 72 hours. Responses for Quinine (**A**), Quinidine (**B**), Clotrimazole (**C**), and Trifluoroperazine (**D**) are shown (with IC50s).

6) *Generate specific antibodies that recognize each of the malaria channel proteins by means of inoculating rabbits and mice with K^+ channel-GST fusion proteins.*

7) *Perform immuno-biochemical and RNA analyses of the temporal and spatial expression pattern of each of the channels in the life cycle of the parasite.*

In order to examine the expression and location of the native channel proteins in malaria parasites we raised polyclonal antisera against soluble portions of PFK1 and PFK2 and affinity purified the antibodies. Immunoblot (Western) of protein from cultured *P. falciparum* showed specific detection of proteins at ~200kD for PFK1 (with higher molecular weight complexes above 250kD) and ~260kD for PFK2 (with large complexes above ~350kD) (**Figure 6**). When cells were synchronized and harvested at specific life stages immunoblots showed that PFK1 was present through out the life cycle of *P. falciparum* in culture. PFK2 showed stage-specific expression with a peak during late trophozoite-to-early schizont stages. These results agree with the staged microarray data for ESTs published by PlasmoDB (**Refs next to last & last**). Immunofluorescence of plasmodium-infected erythrocytes with the same antibodies showed differential staining patterns. PFK1 was more diffuse with peripheral enhancement suggesting localization on the host erythrocyte membrane (**Figure 7**). PFK2 exhibited punctuate staining patterns suggesting localization on the parasite.

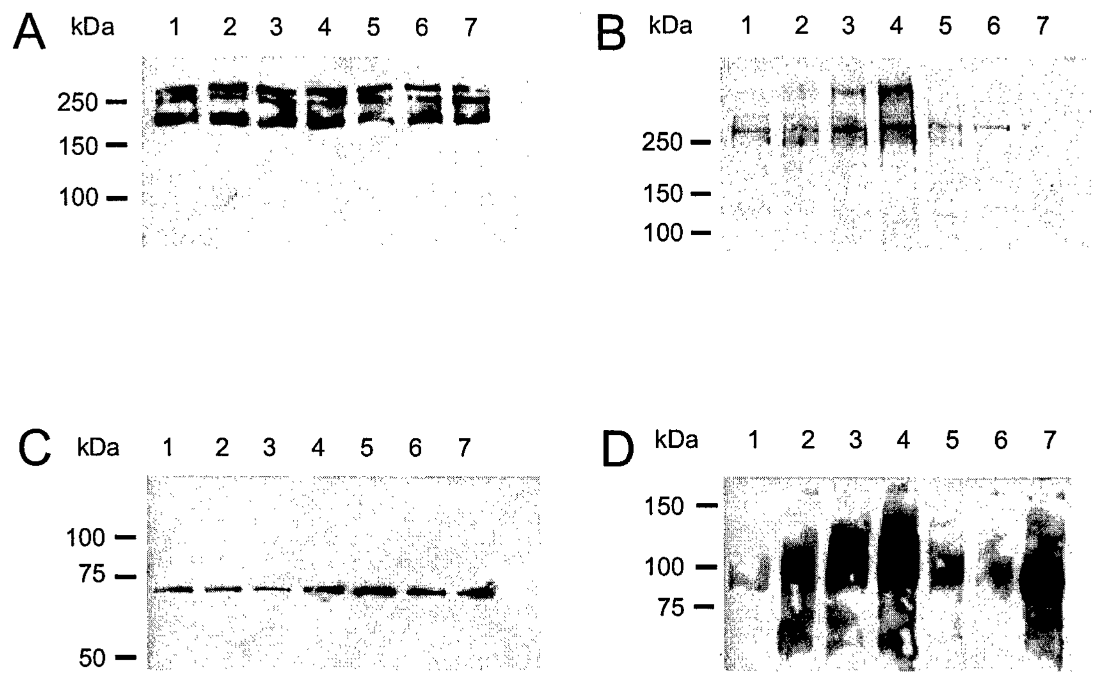

Figure 6. Stage-specific expression of native PFK1 (**A**) and PFK2 (**B**). Synchronized *P.falciparum* harvested at 8 hour intervals over 48 hours. Immunoblot analysis with specific antibodies. Lane 1=87% rings lane 2=80% early trophs; lane 3=97% late trophs; lane 4=35% late trophs & 62% early schizonts; lane 5=74% early rings& 26% late trophs+schizonts; lane 6=90% rings; lane 7=75% rings, 25%earlt trophs. **C**, Immunoblot for heat shock protein and endoplasmic reticulum marker GRP(BiP) showing protein loading control. D, Immunoblot for KAHRP (Knob-associated histidine-rich protein) with known trophozoite/schizont stage-specific expression.

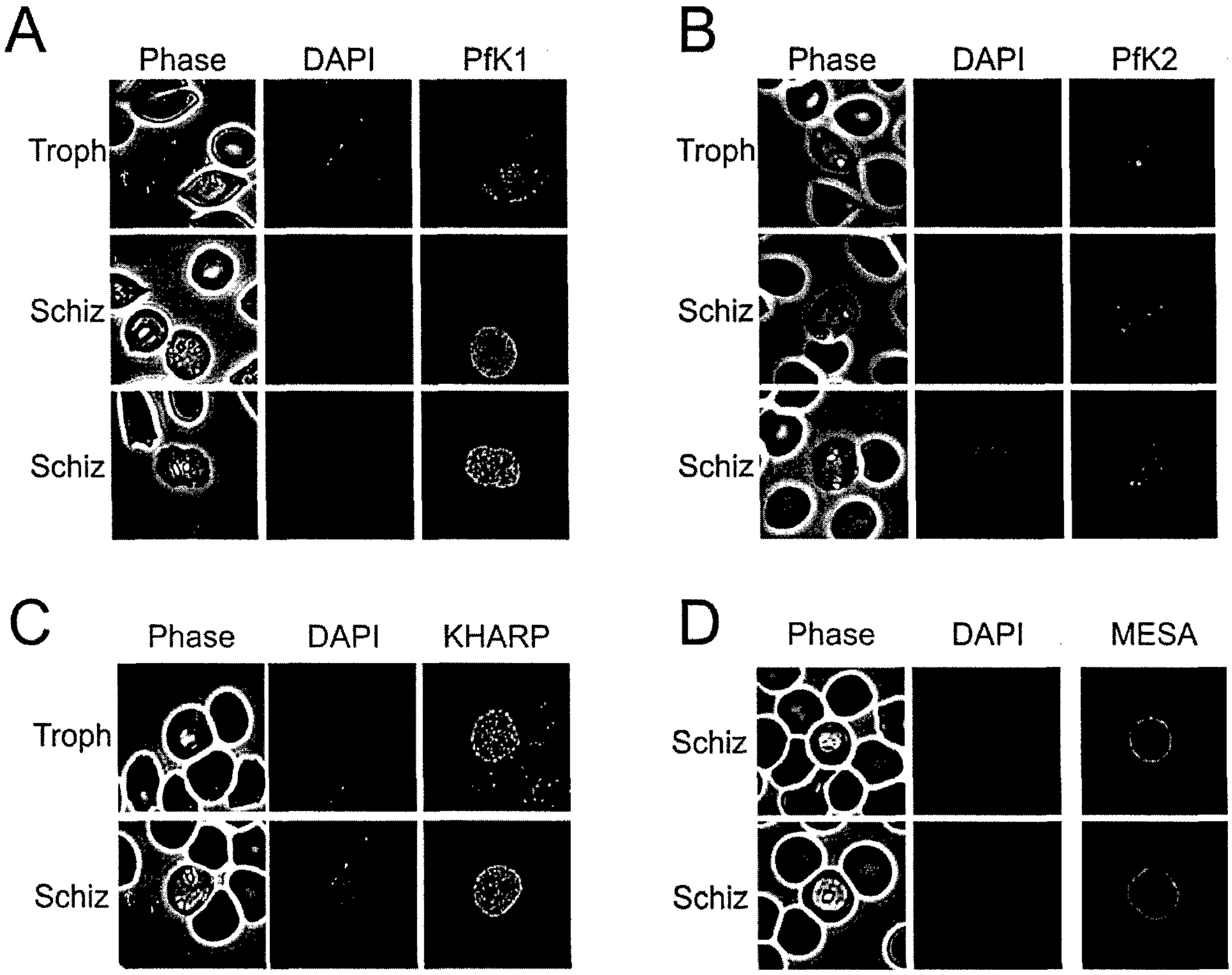

Figure 7. Immuno-localization of PfK1 and PfK1 in parasite-infected erythrocytes. Panels show micrographs of (from left to right) phase contrast, DAPI staining of nuclei, and immunofluorescence of specific malaria-encoded proteins. **A**, PfK1, showing peripheral enhancement suggestive of erythrocyte membrane localization. **B**, PfK2, showing localization on the parasite. **C**, KAHRP which is known to associate with knobs on the surface of the infected erythrocyte membrane. **D**, MESA (Mature parasite-infected Erythrocyte Surface Antigen), which is known to localize diffusely to the host cell membrane.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

- Two K^+ channel gene sequences have been identified and cloned from *Plasmodium Falciparum.*
- Three additional K^+ channel genes identified and cloned from *Leishmania major.*
- Two additional K^+ channel genes identified and cloned from *Toxoplasma gondii.*
- Three additional K^+ channel genes identified and cloned from *Trypanasoma cruzi.*
- Heterologous expression supports K^+ current function of the Malaria and Leishmania channel proteins.
- PFK1 and PFK2 are sensitive to block by chloroquine, quinidine, and TFP.
- Expression of PFK channel protein is verified in *P. falciparum.*
    - Stage-specific protein pattern differences detected for PFK1 and PFK2.
    - Immunofluorescence indicates that PFK1 and PFK2 are differentially expressed on parasite and red blood cell membranes and parasite, respectively.
- Anti-parasite activity of K^+ channel blockers suggests the necessity of PFK channels in parasite survival.
- Projects on-going:
    - Full biophysical characterization of all cloned channels.
    - Further pharmacological testing of all.
    - High-throughput screen development.

REPORTABLE OUTCOMES:

The results described regarding the malaria channels is in manuscript form (see appendix) nearing readiness for submission for publication in peer-review scientific journal.

APPENDICES:

1. Abstract Presented at the 16th Woods Hole Molecular Parasitology Meeting September 2005.

2. Draft of manuscript to be submitted for publication

APPENDIX 1

Molecular and Cellular Characterization of Potassium Channels

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Potassium channels are transmembrane proteins that gate open and closed to regulate the movement of K^+ ions and function to maintain the electrochemical gradients across cell membranes. The potential energy generated by electrochemical gradients is required for such things as nutrient and waste transport, and maintenance of the intracellular chemical milieu required for normal cellular enzymatic functions. These channels are essential for the survival of all known cells types; prokaryotes and eukaryotes alike, and are likely to be critical in allowing various microorganisms to rapidly and efficiently respond to changes in the chemical composition of its extracellular environment. We have used a bioinformatic approach to search the genome databases of various human-disease causing organisms, including *Plasmodium falciparum* and *Leishmania major* Searches were performed with the conserved signature motif GYG, which is located in the pore region of the K^+ channel sequence. Here, we present data on the molecular and cellular elucidation of these various K^+ channels. Additionally, we have commenced the investigation of the electrophysical properties of these proteins. Characterization of the K^+ channels from these infectious organisms and their subsequent specific blockade may generate potential new avenues of chemotherapy against these highly infectious and virulent organisms.

APPENDIX 2

Manuscript Draft.

Differential Expression and Localization of Two Potassium Channels in *Plasmodium falciparum*

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Keywords

Plasmodium falciparum, malaria, potassium channel, electrophysiology, anti-malarial

Abbreviations

PfK1/2, *Plasmodium falciparum* potassium channel 1/2; IFA, Indirect Immunofluorescence Assay; KAHRP, Knob-Associated Histidine Rich Protein; MESA, Mature parasite-infected Erythrocyte Surface Antigen, GRP(BiP), Glucose-Regulated Protein (Binding protein); TBS, Tris-Buffered Saline; PBS, Phosphate Buffered Saline; DAPI, 4',6-Diamidino-2-phenylinodole dihydrochloride; AP, Apamine; CX, Charybdotoxin; QN, Quinine; QD, Quinidine, HD, Haldol; TFP, Trifluoperazine dihydrochloride; TB, Tubocurarine chloride; VR, Verrucolgen; CL, Clotrimazole; BC, Bicuculline methiodide; CQ, chlorquine

Abstract (Max: 250 words) currently 196 words

Potassium channels function in the maintenance of transmembrane electrochemical gradients and are essential to the survival of all known cell types. During its lifecycle, *Plasmodium falciparum* parasites must successfully traverse widely variant environmental milieus, including the mosquito midgut, red blood cell cytosol and the human circulatory system. Therefore, it is likely that expression and function of potassium channels would play an essential role in parasite survival. We report a detailed cellular and biochemical investigation of *P. falciparum* K^+ channels. Two putative K^+ channels genes, *pfk1* and *pfk2*, are encoded in the *P. falciparum* genome. PfK1 is constitutively expressed in all asexual forms of the parasite and is localized to the red blood cell surface in parasite-infected red blood cells. Conversely, PfK2 is expressed only in schizont and merozoite parasite stages and localizes to discrete foci within schizont-infected red blood cells. Response of *P. falciparum* parasites to exposure to various K^+ channel blocking drugs was also investigated using 72 hour [^{3}H]-hypoxanthine incorporation assays. Clotrimazole, bicuculline methiodide, tubocurarine chloride, trifluoperazine hydrochloride and haldol exert an anti-malarial effect on asexual parasites. Characterization of the *P. falciparum* K^+ channels and their subsequent specific exploitation may generate new avenues of anti-malarial chemotherapy.

Introduction

The most severe form of human malaria is caused by infection with the protozoan parasite *Plasmodium falciparum*. The increasing ineffectiveness of many mainstream anti-malarials due to emerging parasite drug resistance, is rapidly urging investigation of potential new molecular targets for future anti-malarial drug development. During its lifecycle, *P. falciparum* parasites must successfully traverse widely variant environmental chemical milieus, ranging from its intracellular locale in the red blood cell, to its extracellular exposure to the human circulatory system and various milieus within the Anopheline mosquito vector, in order to maintain a persistent infection in a human population. The ability of the parasite to rapidly adapt to variations in its environment is essential to its survival. Central to a parasite's survival in the infected red blood cell is the synthesis of parasite-induced new permeation pathways (NPP) (see reviews by Kirk {2001 #17; 2004 #18}). NPP are hypothesized to include the many classes of channels

and transporters encoded by the *P. falciparum* genome. It is thought that these malaria proteins modify the red blood cell to facilitate transport of nutrients, wastes and low molecular mass solutes. Bioinformatic approaches are now being employed to identify the numerous genes in the parasite genome that encode these channels and transporters (reviewed recently by Martin *et al.*, {2005 #16}).

One class of channels, the potassium channels, are transmembrane proteins that gate open and closed to regulate the movement of K^+ ions and thus regulate the electrochemical gradient across the cell membrane. These channels are essential for the survival of all known cells types; prokaryotes and eukaryotes alike. The potential energy generated by electrochemical gradients is essential for maintenance of the intracellular chemical milieu required for normal cellular enzymatic functions. Regardless of origin, K^+ channels share several common features, including membrane topology, with 2, 4 or 6 transmembrane domains, two of which forming the "pore region" through which K^+ ions move (see Fig. 1) and possession of a highly conserved GYG signature motif located within the pore region of the protein (see Hille for review {2001 #14}. Functional channels result from a ternary complex of four identical or closely related subunits, with the pore region being formed by the four-fold symmetry of the component subunits. K^+ channels function to control flow of K^+ ions across cell membranes down electrochemical gradients and thus play essential roles in maintenance of resting membrane potential of cells and intracellular osmolarity. Modes and rate of activation (voltage dependence and Ca^{2+} sensitivity), conductance and sensitivity to K^+ channel blocking drugs vary depending on the class of K^+ channel; Ca^{2+}-activated K^+ channels can be broadly classified into 3 categories; big K^+ (BK), intermediate K^+ (IK) and small K^+ (SK) conductance channels. The Gardos K^+ channel, located in the membrane of the human red blood cell, is an example of an IK channel.

Here, we used a bioinformatic approach to identify two putative K^+ channel genes in the *P. falciparum* genome. Homology searches of the genome were performed using the highly conserved K^+ channel GYG signature motif to extract two genes, designated *pfk1* and *pfk2*. We have conducted a detailed investigation of the expression and cellular

localization of both PfK1 and PfK2. We have shown that PfK1 is expressed in all asexual parasite stages, whereas PfK2 is expressed only in late schizonts and merozoites. Detailed IFA studies performed on synchronized *P. falciparum* cultures demonstrated the differential cellular localization patterns of these K^+ channels. PfK1 is localized to the red blood cell surface of asexual stage parasites, whereas PfK2 is localized to schizont and merozoite stage parasites. The data presented here is the first detailed elucidation of K^+ channels in *P. falciparum* at the cellular level. Additionally, a series of [^{3}H] radio-label incorporation assays were used to investigate parasite sensitivity to various K^+ channel blocking compounds. The identification of only two putative K^+ channels encoded within the *P. falciparum* genome, in conjunction with their very different expression and localization profiles within the parasite, suggest that these genes are extremely important to maintenance of parasite viability. The rationale that K^+ channels are essential for parasite survival is central to our research hypothesis, and that the subsequent potential blockade or disruption of parasite K^+ channels could provide a novel avenue for anti-malarial therapeutic development.

Materials and Methods

Parasite Culture – *Plasmodium falciparum* 3D7 parasites were maintained in *in vitro* culture using standard cultures conditions {Trager, 1976 #3} supplemented with 0.5% (w/v) Albumax II (Invitrogen, Carlsbad CA). Highly synchronous parasite cultures were obtained using successive rounds of sorbitol lysis {Lambros, 1979 #30}. Percentage parasitaemias and differential cell counts of culture samples were determined by microscopic examination of Giemsa stained smears.

Homology Searches and Molecular Cloning – Homology searches of the *P. falciparum* genome database (www.plasmodb.org) were performed using the conserved K^+ channel GYG signature motif. Two gene sequences, designated *pfk1* (PFL1315w) and *pfk2* (PF14_0622) were extracted. The genes encoding *pfk1* and *pfk2* were amplified from genomic *P. falciparum* 3D7 DNA by PCR using specific oligonucleotide primers (Table 1) and cloned into standard laboratory *Escherichia coli* plasmids. All nucleotide sequences were confirmed by automated sequence analysis (Genewiz Inc.,

Great Neck NY). The nucleotide sequences of *pfk1* and *pfk2* were deposited in GenBank with the accession numbers **XXX** and **XXX**, respectively.

RT-PCR analysis was performed……

Antisera Production, Western Blotting and Indirect Immunofluorescence Assays (IFA) – Polyclonal rabbit antisera were raised against recombinant regions of PfK1 and PfK2. Various regions of *pfk1* and *pfk2* were cloned into the *E. coli* protein expression vector pGEX-KG {Guan, 1991 #1}. GST fusion proteins were expressed in *E. coli* BL21(DE3) cells (Novagen Inc., Milwaukee WI) and purified using standard affinity chromatography techniques {Bennett, 1997 #2}. Antibodies were raised in female New Zealand White rabbits by injecting 125 μg of purified protein emulsified in Freund's complete or incomplete adjuvant (Covance, Princeton NJ). Antibodies were affinity purified from whole rabbit serum. Briefly, 1 mg of purified GST-PfK1 or -PfK2 fusion proteins were coupled to pre-swelled CN-Br Sepharose (Amersham Biosciences, Buckinghamshire UK) in 25 mM HEPES pH 8.5, 500 mM NaCl for 2 hours at room temperature. Gel was then washed (TBS: 100 mM Tris-HCl, pH8.0, 500 mM NaCl) and mock eluted with 200 mM glycine prior to overnight incubation with polyclonal rabbit antiserum. Gel was then washed twice in TBS and affinity purified antibodies eluted with 200 mM glycine directly into 1 M Tris-HCl, pH8.0. Purified antibodies were used for further immunologic analyses of PfK1 and PfK2.

Lysates were prepared for Western Blotting from *P. falciparum* 3D7 parasites synchronized by three successive rounds of sorbitol lysis. Cultures were sampled at 8 hourly intervals and the cells lysed using 0.15% (w/v) saponin. Parasites were washed in STE (100 mM NaCl, 20 mM Tris-HCl pH 8.0, 50 mM EDTA), before resuspension in reducing SDS-PAGE loading buffer and incubation at 37 °C for 30 minutes. Samples were not boiled to facilitate increased solubility of transmembrane proteins when analyzed by SDS-PAGE. Prepared lysates were resolved in 7.5% or 4–15% gradient polyacrylamide gels (Bio-Rad Laboratories, Hercules CA) before transfer to nitrocellulose membranes. Western blotting was performed using affinity purified anti-

PfK1 F3 or -PfK2 F4 antibodies. Blots using the control antisera polyclonal anti-KAHRP (raised against a 6 x His-C-terminal fragment of KAHRP fusion by Karena L. Waller, Monash University, Australia; unpublished data; provided by Ross L. Coppel) and anti-GRP(BiP) ({Kumar, 1991 #4}; MRA20, MR4 ATCC Manassas VA) were also performed. Western blots were detected using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston MA).

Cellular localization of PfK1 and PfK2 was examined by Indirect Immunofluorescence Assays (IFAs). IFAs were performed on *P. falciparum* 3D7 parasites synchronized by sorbitol lysis. Briefly, thin blood smears were prepared from cultures at approximately 12 hourly intervals during the 48 hour asexual lifecycle. Cells were fixed to glass slides using 90% (v/v) methanol / 10% (v/v) acetone for 5 minutes at room temperature before air drying. Aliquots of affinity purified primary antibodies were diluted (1/100 or 1/250) in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 pH 7.4) containing 0.5% (w/v) BSA and applied for 30 minutes. Slides were then washed extensively with PBS, the secondary goat anti-rabbit Alexa Fluor® 488 conjugate antibody (1/1000 dilution; Molecular Probes, Eugene OR) applied prior to staining the parasite genome with 4',6-Diamidino-2-phenylinodole dihydrochloride (DAPI; 10 μg/ml). Slides were washed extensively prior to mounting in Fluoromount-G (Southern Biotech, Birmingham AL) under a glass coverslip. Slides were visualized using an Olympus IX81 electronically motorized microscope (60X objective, N.A. 1.4 combined with 1.6X booster lens and filters appropriate for DAPI, FITC and phase microscopy) and images captured using a Sensicam QE cooled CCD camera (The Cooke Corporation, Romulus MI) and IP Lab Spectrum Scientific Imaging Processing Software (version 3.6.1; Scanalytics Inc., Fairfax VA). Images were then deconvolved using Vaytek Image (version 7.0.76; Vaytek Inc., Fairfield IA) and cropped for presentation. The primary antibodies and dilutions used in these studies were: affinity purified anti-PfK1 F4 (1/250), anti-PfK2 F4 (1/100), polyclonal rabbit anti-KAHRP (1/250; and polyclonal rabbit anti-MESA (1/250; {Bennett, 1997 #2}; provided by Ross Coppel, Monash University, Australia).

Drug Assays – 72 hour [^{3}H]-hypoxanthine incorporation assays were performed to determine the effect of several known K^+ channel blockers on *P. falciparum* growth. These assays were performed in accordance with previously described protocols {Waller, 2003 #6; Fidock, 1998 #5}. Briefly, parasites (0.8% parasitaemia, 3.5% haematocrit) were plated into wells of a 96 well plate containing an equal volume of serial drug dilutions. Plates were incubated for 48 hours under normal parasite culture conditions, prior to the addition of [^{3}H]-hypoxanthine (Amersham Biosciences, Buckinghamshire UK). Plates were harvested at 72 hours onto filter mats using a 96 well plate cell harvestor (Tomtec Inc., Hamden CT) and scintillation counting performed using a Wallac1450 Microbeta Trilux Microplate Liquid Scintillation Counter and Luminometer (PerkinElmer Life And Analytical Sciences, Inc., Boston MA). Drugs included in the study were: Apamine (AP; from Bee Venom), Charybdotoxin (CX; recombinant form of scorpion venom *Leiurus quinquestriatus*; Calbiochem, San Deigo CA), Quinine (QN), Quinidine (QD), Trifluoperazine dihydrochloride (TFP), Tubocurarine chloride (TB), Verrucolgen (VR; from *Penicillium verruculosum*), Clotrimazole (CL), Bicuculline methiodide (BC; Sigma-Aldrich, St Louis MO) and Haldol (injectable; American Pharmaceutical Partners, Schaumburg IL). IC_{50}s and IC_{90}s ± Standard Error of the Mean (SEM) were determined from drug response data using GraphPad Prism v4.00 (GraphPad Software, Inc.).

Microscopic examination of Giemsa stained slides prepared from samples identical to the [^{3}H] growth curve samples during the assay period was used to determine the morphological appearance of parasites at each drug concentration. Differential parasite counts were also performed on greater than 500 total cells for each drug dilution.

Results

Identification of pfk1 and *pfk2*

Homology searches of the *P. falciparum* genome database (www.plasmodb.org) with the conserved signature GYG motif common to many K^+ channels yielded two putative genes, designated here *pfk1* (PFL1315w, as known as *pfkch1* {Ellekvist, 2004 #12}) and

pfk2 (PF14_0622). The *pfk1* gene is approximately 6.1kb in size and is located on chromosome 12, whereas *pfk2* is approximately 4.4kb and located on chromosome 14. Both genes encode proteins that have predicted membrane spanning regions (as indicated by Kyte-Doolittle hydrophilicity plots {Kyte, 1982 #23} of the encoded protein sequences), and a putative pore region (Fig. 1A). Alignments of the pore region protein sequence from PfK1 and PfK2 with other K^+ channels demonstrates overall similarity between the proteins **(Fig 1E).....**

A recent review by Martin *et al.,* {, 2005 #16}, in addition to identifying *pfk1* and *pfk2* as putative *P. falciparum* K^+ channels, also suggested the presence of a possible third putative K^+ channel (PF14_0342). At the time these investigations were performed, we excluded PF14_0342 from analysis, based on the lack of an encoded conserved GYG motif within this gene.

Analysis of PfK1 and PfK2 Expression in Asexual Parasites

RT-PCR analysis of asynchronous *P. falciparum* 3D7 parasites will be performed....

Synchronized *P. falciparum* 3D7 parasites obtained by two successive rounds of sorbitol lysis were used to generate 8 hourly time point samples of parasites throughout the 48 hour asexual life cycle. Approximately equal numbers of parasites (as determined by microscopy and differential cell counts of Giemsa stained smears; Table 2) were loaded for each time point into polyacrylamide gels. Western blots (Fig. 3) were performed using affinity purified antisera specific for PfK1 F3 and PfK2 F4 (locations of recombinant protein fragments used to generate these antisera are indicated in Fig.1). Our blots showed PfK1 expression, detected as a group of three high molecular mass bands of between 200 and 300 kDa, was approximately equal throughout the 48 hour asexual lifecycle (panel A). The detection of multiple bands for PfK1 may be attributed to a functional channel resulting from a multimer of PfK1 subunits that were not adequately disrupted during the relatively mild sample preparation conditions. Conversely, PfK2 expression was demonstrated to alter throughout the 48 hour time course, with maximal expression being detected in samples rich in merozoites and late

schizonts (mature schizonts contain many developing merozoites each; panel B). Control western blots were performed simultaneously using antibodies against the endoplasmic reticulum marker protein Glucose-related protein GRP(BiP) (gene PF10875w in www.plasmodb.org; panel C; {Kumar, 1991 #4}), that has previously been shown by microarray to have constitutive expression across the 48 hr asexual parasite life cycle {Le Roch, 2004 #8; Ben Mamoun, 2001 #10; Bozdech, 2003 #11} The increased levels of GRP(BiP) detected in samples taken later in the time course, is likely due to upregulation during stress conditions caused by cultures having been maintained at high parasitaemia for 48 hours. The stage specific parasite antigen Knob Associated Histidine Rich Protein (KAHRP) was visualized in mature trophozoite and schizont samples, but not in ring rich culture samples.

Whole Cell Localization of PfK1 and PfK2 by Indirect Immunofluorescence Assays (IFA)

Highly synchronous *P. falciparum* 3D7 cultures were used to make thin blood smears at 12 hourly intervals during the 48 hour asexual life cycle. Indirect Immunofluorescence Assays (IFAs) were then performed to localize PfK1 and PfK2 within the parasite-infected red blood cell using affinity purified anti-PfK1 F4 and –PfK2 F4 antibodies. Control IFAs using polyclonal anti-KAHRP and anti-MESA antisera {Bennett, 1997 #2} were also included. Figure 4 shows representative IFAs of ring-, trophozoite- and schizont-infected red blood cells. Although detectable by western, PfK1 was not detected by IFA in ring-infected red blood cells. Labeling was however detected in trophozoite- and schizont-infected red blood cells. The level of labeling increased with parasite maturity and was located on the surface of the infected red blood cell, in a semi-punctuate localization pattern (Fig. 4A). Similar IFA results were obtained for PfK1 localization when either anti-PfK1 F3 or F4 antibodies were used. IFAs performed with anti-PfK2 F4 antibodies also failed to label ring-infected red blood cells. Minimal labeling was detected in trophozoite-infected red blood cells. Intense punctuate labeling of PfK2 was detected in mature schizont infected red blood cells, in such a pattern as to suggest co-localization with the developing merozoites in the schizont (Fig. 4B).

Control IFAs were performed with anti-KAHRP and anti-MESA antisera (Fig. 4C and D). Anti-KAHRP IFAs show no detectable reactivity to ring-infected red blood cells. Increasing reactivity was observed with increasing maturation age of the parasite, with maximal labeling detected on the red blood cell membrane surface of trophozoite and schizont-infected red blood cells. KAHRP is expressed by the intracellular parasites and is exported into the red blood cytosol where it associates with the red cell proteins spectrin and actin, resulting in the formation of electron-dense, knob-like structures located at intervals on the red cell membrane surface (see Cooke *et al.,* for a recent review {Cooke, 2001 #20}). Anti-MESA IFAs showed no detectable labeling of ring-infected red blood cells. Increasing levels of labeling were observed for trophozoite- and schizont infected red blood cells. MESA was observed at the surface of the mature parasite-infected red blood cell and had a less punctuate, almost confluent appearance. MESA is a parasite-encoded protein that is exported from the intracellular parasite into the red blood cell cytosol where it associates with the red blood cell membrane skeleton protein, protein 4.1 but has no specific association with knobs (see Cooke *et al.,* for a recent review {Cooke, 2001 #20}).

Additional IFA experiments are currently underway to further define the localization of PfK1 and PfK2 within the parasite-infected red blood cell. We have generated a set of unique anti-PfK1 and –PfK2 mouse antibody reagents to enable co-localization IFA experiments to be performed in conjunction with several other anti-parasite protein (rabbit) antibodies.

Response of *Plasmodium falciparum* to Potassium Channel Blocking Compounds

72 hour [^{3}H] hypoxanthine incorporation assays were performed to determine the anti-malarial effect of various compounds that have K^+ channel blocking activity on the growth of *P. falciparum* parasites. The established anti-malarials Quinine (QN) and Quinidine (QN) were examined, in addition to Apamine (AP), and Bicuculline methiodide (BC), Charybdotoxin (CX), Clotrimazole (CL), Haldol (HD), Trifluoperazine hydrochloride (TFP), Tubocurarine chloride (TB) and Verrucolgen (VR). Specific activities and clinical uses of each compound are shown in Table 3. IC_{50}s and IC_{90}s ± standard error of the

mean (SEM) were determined (Table 3). Graphical plots of the growth inhibition data for the drugs are shown in Fig. 5. Three compounds showed no detectable parasite growth inhibition in initial assays within the concentration ranges used: AP (0 – 500 nM), CX (0 – 250 nM) and VR (0 -1500 nM), and were therefore excluded from further analysis (data not shown). This investigation is the first report of a lack of an anti-parasitic effect for apamine and verrucolgen on *in vitro P. falciparum* cultures, whereas the lack of an effect of CX on malaria-infected cells has been previously reported {Kirk, 1992 #27}. Additionally, our assays determined the IC_{50} and IC_{90} for TFP, BC, HD and TB (Table 3).

Discussion

During the lifecycle of *P. falciparum,* parasites are exposed to widely variant environments. Essential to the maintenance of a persistent infection in the human population, is their successful ability to rapidly adapt to these environments. In asexual stage parasites, many proteins are expressed by the intracellular parasite, that are exported into the red blood cell where they subsequently modify red blood cell structure and function (see Cooke *et al.,* for a recent review {Cooke, 2001 #20}). As part of this red blood cell modification, parasite-encoded new permeation pathways (NPP), that facilitate the transport of low molecular mass solutes, are synthesized (reviewed in {Kirk, 2001 #17; Kirk, 2004 #18}). These NPP are likely to include many of the parasite-encoded channels and transporters that are being identified in the genome by bioinformatics searches (see Martin *et al.,* for a recent review {Martin, 2005 #16}).

Here, we present the first detailed cellular investigation detailing the the differential expression and localizations of two *P. falciparum* potassium channels, PfK1 and PfK2. We generated a set of protein-specific antisera to facilitate analysis of PfK1 and PfK2 protein expression throughout the asexual lifecycle, via western blotting and IFA. Data obtained from western blots demonstrated that PfK1 is expressed throughout the entire 48 hr asexual cycle, whereas as IFA was only able to detect PfK1 expression from early trophozoite- through schizont-infected red blood cells. Our western blot data is in complete accord with previous studies of PfK1 by mRNA microarray and Real Time

PCR ({Ben Mamoun, 2001 #10; Bozdech, 2003 #11; Le Roch, 2004 #8; Ellekvist, 2004 #12}) but we were unable to localize PfK1 in immature ring-stage parasites via IFA. The density of PfK1 channels in rings may be too low, such that their detection was beyond the limit of resolution by IFA. In support of this argument, more intense labeling was detected in more mature-infected cells (Fig. 4), and also in cells that were multiply infected (data not shown). Additionally, failure to detect PfK1 in rings may also be attributed to unavailability of the reactive epitope. Even though the same affinity purified anti-PfK1 F4 antibodies were used for both western and IFA studies, it is possible that the F4 epitope is "revealed" in the denatured western samples, but that in IFA, the F4 epitope may be obscured while in transit to its final location at the red blood cell membrane. Our western blot and IFA data for PfK2 expression is also in agreement with previous studies performed at the RNA level {Ben Mamoun, 2001 #10; Bozdech, 2003 #11; Le Roch, 2004 #8}, with PfK2 being expressed in asexual stages rich in merozoites (late schizonts). The western blot demonstrated a 'tapering off' effect for PfK2 expression, which may be attributed to some carry over of PfK2 protein by the invading merozoite into the newly formed ring. Low levels of PfK2 labeling were detected by IFA in some young ring stage parasites; labeling was confined to the parasite, and did not appear to be present in the red blood cell cytosol or membrane surface (data not shown).

Our IFA data strongly indicates a red cell membrane surface location for PfK1. At this location, we hypothesize that PfK1 is expressed by the parasite and exported into the red blood cell, where it is inserted into the red blood cell membrane. Analysis of the PfK1 protein sequence for motifs indicated as potential export/transport signals was performed by the Haldar group, using their optimized consensus motif RxSRILAExxx predicted by Multiple Expectation Maximization for Motif Elicitation (MEME) (detailed in {Lopez-Estrano, 2003 #22}{Hiller, 2004 #21}. Manual searches of PfK1 sequence for this motif yielded two potential low-value plasmoHT sequences, FFYRKLKNTFM and NFRRFLSSYKS (located 99 and 169 residues from the N-terminal end of PfK1, respectively). PfK1 also contains an SS sequence that has been predicted in several studies to be a cleavable endoplasmic-reticulum signal sequence facilitating entry into

the parasite's secretory pathway **(finer look at references!!!).** Therefore, these sequences may function as export signals, directing PfK1 for export into the red blood cell, although this has not yet been experimentally tested (K. Haldar, unpublished data). Contrary to PfK1, our western and IFA data for PfK2 appears to be supportive that PfK2 is localized with the merozoite. At this location it is not clear if PfK2 is a traditionally exported protein, since it may only be trafficked as far as the merozoite plasma membrane. Analysis of the PfK2 sequence by the Haldar group {Hiller, 2004 #21} indicated the presence of a low value plasmoHT motif YRRKNVLNIFN (located 78 residues from the N-terminus) and the presence of a possible SS cleavage motif, although its configuration and location make it unclear as to whether these may actually function in PfK2 export (K. Haldar, unpublished data). **(must check this bit over with Haldar group for accuracy and ability to include).** Further exploitation of our unique set of protein specific reagents in IFA experiment may help elucidate the trafficking of PfK1 and PfK2 within the parasite.

The semi-punctate pattern of PfK1 labeling is reminiscent of that observed with the anti-KAHRP antibodies that label the knob-like protrusions on the red blood cell surface (Fig. 4C). Experiments to determine if PfK1 co-localizes with KAHRP at knobs could not be performed during this investigation, as both the anti-KAHRP and anti-PfK1 F4 were generated in rabbits. The generation of anti-PfK1 antibodies in mouse are underway, so as to allow for more refined co-localization experiments to be performed. Similarly, continued investigation of PfK2 at the IFA level will be performed using anti-bodies that co-localize to the merozoite to further define its possible location within the merozoite. An additional avenue of investigation that may help further elucidate the location of PfK1 and PfK2 would be the identification of any protein interacting partners. Since both of these proteins are predicted to be transmembrane, it is possible that they interact with other proteins within membrane or membrane skeleton of the red blood cell or merozoite. Also, in many other cell types K^+ channels have associated accessory proteins that are critical for their function, for example the human ether a-go-go (HERG) K^+ channel and its accessory protein mink **(reference).** Possible future protein

interaction experiments may help identify any interacting or accessory proteins to PfK1 and PfK2.

Protein expression data presented here, in addition to previous RNA data {Ben Mamoun, 2001 #10; Bozdech, 2003 #11; Le Roch, 2004 #8; Ellekvist, 2004 #12} demonstrates that PfK1 and PfK2 expression are spatially separated in both time and location in the parasite. PfK1 is expressed in all asexual stage parasites on the red blood cell membrane surface (and at lower levels in gametocytes {Le Roch, 2004 #8}), whereas PfK2 is only expressed in merozoites (and in late schizonts) and appears to be localized to the developing merozoite. The fact that the parasite encodes at least two K^+ channels (a third putative K^+ channel gene (PF14_0342 containing a GKG motif instead of GYG) was indicated in searches detailed by Martin *et al.,* {Martin, 2005 #16}, but was not extracted as a putative K^+ channel using our GYG search criteria) that are differentially expressed at various stages of parasite development, strongly indicates to a stage specific importance for each gene product. PfK1, with its location on the red blood cell membrane surface and expression throughout the asexual lifecycle (in addition to gametocytes and sporozoites) is likely to be essential for intracellular survival, maintenance of red blood cell integrity, electrochemical gradient, membrane potential **(that paper of membrane potential of IRBCs by Kirk)** and the composition of the intracellular milieu in response to changes induced to the red blood cell by the developing parasite. Conversely, PfK2 with its expression limited to merozoites stages (PfK1 is also expressed in merozoites), is likely to be essential in the maintenance of the electrochemical gradient and membrane potential in extracellular merozoite stages that are exposed to the human circulatory system environment and may act in concert with PfK1 during this time to overcome the extreme environment change the parasites are exposed to when released from the rupturing schizont. The putative third K^+ channel, PF14_0342 is expressed in all asexual stage parasites and gametocytes, but not sporozoites (according to online microarray data {Bozdech, 2003 #11; Le Roch, 2004 #8}, and if formally proven to be a K^+ channel, may act in concert with PfK1 in asexual stages and gametocytes, and with PfK2 in merozoites. Currently, we have parasite transfection experiments underway to examine the likely essential nature of K^+

channels to the survival of the parasite *in vitro* via genetic manipulation of *pfk1* and *ofk2*. We have obtained faint PCR data to demonstrate the presence of knockout and knockdown PfK1 and PfK2 parasites in transfection cultures (data not shown), but despite several repeated attempts, we have been unable to isolate a PfK1 or PfK2 knockout or knockdown clone. The fact that we can detect integration via PCR in cultures, but not isolate a clone suggests that upon disruption of *pfk1* or *pfk2*, a lethal phenotype results, and the parasites die out before the end of the limiting dilution cloning protocol (K.L. Waller, unpublished observations). These data support our hypothesis that pfk1 and pfk2 and their expression are essential for parasite viability.

...........draw a cool cartoon here of expression in different stages of parasites to help reader relate to differential expression, and the likely importance of each gene at different stages.

Response of asexual parasites to exposure to various K^+ channel blockers was examined by 72 hr [^{3}H] hypoxanthine incorporation assays. In our assays, the IC_{50} and IC_{90} data obtained for the anti-malarials QN and QD against *P. falciparum* 3D7 was in agreement with that reported for other strains of *P. falciparum* parasites {Sidhu, 2002 #24}. Clotrimazole, an anti-fungal agent that blocks IK type Ca^{2+}-activated K^+ channels, conferred an $IC_{50} = 0.60 \pm 0.01$ μM and $IC_{90} = 0.80 \pm 0.01$ μM in our assays. Previous studies had also determined that clotrimazole exerts an anti-malarial effect on asexual stages *in vitro* {Saliba, 1998 #25; Tiffert, 2000 #26}. Those studies demonstrated an IC_{50} of between 0.2 and 1.1 μM, depending on the strain of parasite used. Our data is in accordance with these previous findings. Similarly, microscopic examination of Giemsa stained slides prepared from samples identical to the [^{3}H] growth curve samples during the assay period corroborated (data not shown) the profound morphological effects of CL previously noted against mature stage parasites {Tiffert, 2000 #26}. We observed total death of all mature stage parasites at [CL] > 3.1 μM, with survival of immature ring forms at [CL] ≤ 3.1 μM.

Trifluoperazine dihydrochloride (TFP), a potent calmodulin and dopamine receptor antagonist, has previously been noted to have an inhibitory effect on invasion and parasite maturation in the Brazilian *P. falciparum* 7G8 line and FCR-3 strain {Tanabe, 1989 #29; Matsumoto, 1987 #28}. Tanabe and co-workers used parasite viability determined via microscopic examination of stained smears to ascertain a TFP IC_{50} of 13 $\pm$ 0.7 μM after 72 hours culture (*P. falciparum* FCR-3 strain) {Tanabe, 1989 #29}. In our experiments, we determined a modestly higher IC_{50}=44.99 $\pm$ 1.0 μM for TFP, which may in fact be attributed to differences between the *P. falciparum* strains / isolates examined. The phenomenon of variable IC_{50} levels depending on the parasite strain / isolate used in *in vitro* experiments has been extensively noted in the literature **(XXX).** For example, clotrimazole IC_{50}s varied from between 0.2 and 1.1 μM depending on parasite background, in a study that used chloroquine (CQ) resistant, mildly CQ resistant and CQ sensitive parasites {Saliba, 1998 #25; Tiffert, 2000 #26}. For each of the non-mainstream drugs we examined for potential anti-malarial activity, more extensive studies would need to be performed to determine the IC_{50} range across many diverse parasite genetic backgrounds.

To our knowledge, this is the first report of the *in vitro* anti-malarial effect of BC, HD and TB. Bicuculline methiodide (BC) is a water soluble derivative of bicuculline, an isoquinoline alkaloid (isolated from the *Dicentra cucullaria* plant) that blocks Ca^{2+}-activated SK type K^+ channels and is a $GABA_A$ receptor antagonist. In our assays, BC conferred an IC_{50} = 79.33 $\pm$ 2.9 μM, and resulted in death of all mature stage parasites at [BC**] > XXX**, with survival of immature ring forms at [BC] **< XXX** . Although BC achieved a relatively low IC_{50} when tested against asexual *in vitro P. falciparum* cultures, it may not translate to being a useful human anti-malarial. BC causes convulsions and seizures, and thus may be detrimental to malaria-infected humans, especially if the IC_{50} required to kill parasites is greater than the therapeutic dose used to induce convulsion.

Haldol, a butyrophenone clinically used as an anti-psychotic drug is also a dopamine receptor antagonist that conferred an IC_{50} = 4.29 $\pm$ 0.17 μM. It is unlikely that haldol

confers a therapeutic value as an anti-malarial as maximal therapeutic plasma doses recommended for treatment of psychosis is 25 μg per liter, or 0.118 μM [HD].
Morphology of parasites on slides.....800 slides to go!!

Tubocurarine chloride (TB), a nicotinic acetylcholine receptor antagonist and non-depolarizing muscle relaxant, isolated from curare, an extract from the South American jungle plant *Chondodnedron tomentosum* and formerly used as arrow poison by South American Indians, conferred an IC_{50}=129.01 ± 3.2 μM. Due to the extreme toxic nature of TB, it is unclear as to weather TB could ever be exploited as a potential anti-malarial.
Maximal therapeutic dose??? Morphology of parasites on slides.....

Apamine, charybdotoxin and verrucolgen were each characterized as having no anti-parasitic activity in the 72 hour *in vitro* assays performed. This is the first report for lack of effect for apamine and verrucolgen, whereas charybdotoxin (an inhibitor of Ca^{2+}-activated and voltage-gated K^+ channels) had previously been elucidated as having no effect on $K^+(^{86}Rb^+)$ transport in parasite-infected red blood cells {Kirk, 1992 #27}. Our charybdotoxin data supports their previous observations.

The screening of these alternate compounds for anti-malarial activity was prompted not by a desire to turn these compounds into possible human therapies, but to help elucidate the pharmaco-dynamic properties of the K^+ channels in the malaria-infected red blood cell. Different types of Ca^{2+}-activated K^+ channel (BK, IK or SK) are blocked by different compounds. Using these compounds in assays with infected cells may provide additional information as to which type of K^+ channel PfK1 and PfK2 belong to. Charybdotoxin and Iberiotoxin (also a scorpion toxin) are both active (in the nM range) against BK channels, clotrimazole (nM) is active against IK channels and Apamine (nM) and Curare (μM; the parent compound for Tubocurarine chloride) are active against SK type channels (reviewed in {Vergara, 1998 #31}. In our assays, we showed no activity for apamine, but activity for tubocurarine chloride, thereby suggesting the presence of SK-type K^+ channel activity, but which is apamine-insensitive. Clotrimazole, an IK type channel blocker showed activity, whereas Charybdotoxin, a BK type channel blocker,

was inactive, thereby suggesting the presence of an IK channel and absence of a BK channel in malaria-infected red blood cells. Closer examination of the amino sequences of PfK1 and PfK2 suggested the PfK1 was most similar to BK type channels, whereas PfK2 as was similar to IK type channels **(data not shown)**, which would be supported by the observance of clotrimazole activity in our assays. Conversely, the BK blocker charybdotoxin did not show activity, possibly indicating that if PfK1 is a BK type channel, it is charybdotoxin insensitive. Further experiments are under way to provide additional information as to the pharmacodynamic and electrophysiological properties of PfK1 and PfK2.

The normal human red blood cell IK type Ca^{2+}-activated K^+ channel, the Gardos K^+ channel, was first described in 1958 {Gardos, 1958 #33} and cloned for electrophysiological analysis by Takahiro and co-workers {Ishii, 1997 #35} . This channel is located in the red blood cell membrane and is blocked by inhibitors such as clotrimazole {Alvarez, 1992 #32} and charybdotoxin {Brugnara, 1995 #34}. (Though traditionally thought of as a BK type channel blocker, charybdotoxin is active against the IK Gardos channel). In our *in vitro* assays, we demonstrated that charybdotoxin was not active in killing malaria parasites. This lack of activity exits even though charybdotoxin is active against the endogenous Gardos K^+ channel of red blood cells. Conversely clotrimazole, a lipophilic compound that can penetrate cell membranes, inhibits the Gardos K^+ channel with an IC_{50} of 0.05 μM {Alvarez, 1992 #32}. We determined a smilar $IC_{50} = 0.60 \pm 0.01$ μM when using malaria-infected red blood cells. Whether the anti-malarial activity of clotrimazole results from direct blockade of the parasite's K^+ channels, or results from altered physiology of the clotrimazole-treated, Gardos channel-blocked red blood cell causing parasite death, is difficult to determine. Further investigations would be required.....

In this study, we have provided a detailed description of the expression and localization of two K^+ channels encoded in the *P. falciparum* genome, and commenced the elucidation of their pharmacodynamic properties. The differential expression and

localization profiles of PfK1 and PfK2, combined with our genetic manipulation observations, strongly support a critical role for these K^+ channels at their different expression stages in maintaining the viability of asexual stage *P. falciparum* parasites. Continued investigation of the pharmacodynamic and electrophysiological properties of these channels in heterologous systems will facilitate our increased understanding of how these channels function. Ultimately, investigations like these may provide a useful avenue for exploitation in the future development of new anti-malarial therapies.

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References

Figure and Table Legends

Figure 1 – PfK1 and PfK2. A, B. Kyte-Doolittle hydrophilicity plots {Kyte, 1982 #23} of the predicted amino acid sequence encoded by *pfk1* (panel A) and *pfk2* (panel B), respectively. Negative deflections signify hydrophobic sequences. Underlying brackets show predicted membrane-spanning segments, 'p' indicates the putative pore region. Protein sequences recombinantly expressed and purified as GST fusion proteins for generating the polyclonal rabbit antibodies used in western blots and IFA, are indicated by broken-line bracketed regions. **C.** Schematic of the membrane topography of a

single subunit of a Ca^{2+}-activated K^+ channel. **D.** Schematic of the tetrameric K^+ channel assembled in the cell membrane. **E.** Alignments of pore region protein sequence from PfK1 and PfK2 with other K^+ channels......

Fig. 2 – RT-PCR. Expression of *pfk1* and *pfk2* was demonstrated from asynchronous *P. falciparum* 3D7 cultures by RT-PCR.

Fig. 3 – Expression of PfK1 and PfK2. Highly synchronous *P. falciparum* 3D7 cultures were sampled every 8 hours during the 48 hour asexual lifecycle and probed by Western Blot. Approximately, 5.2 – 8.9 x 10^6 parasites were loaded per lane. The differential parasitaemias for each sample are listed in Table 2. **A.** PfK1 was detected as a triplet of bands ranging in molecular mass from about 200 – 300 kDa using affinity purified anti-PfK1 F3 antibodies. Approximately equal levels of expression of PfK1 were detected across each sample. **B.** PfK2 was detected as a band of approximately 300 kDa using affinity purified anti-PfK2 F4 antibodies, with maximal expression being detected in samples rich in late schizonts (ie. samples rich in developing merozoites). **C.** Constitutive expression of the heat shock protein and endoplasmic reticulum marker GRP(BiP) across the 48 hr lifecycle of *P. falciparum, detected* at approximately 70 kDa. Increased levels of GRP(BiP) in later samples is likely due to culture stress and increased expression of GRP(BiP), a heat shock protein. **D.** Stage specific expression of KAHRP, observed as a broad band of reactivity at ~100 kDa, with maximal expression observed in mature trophozoite and schizont samples. Minimal expression was detected in culture samples rich in immature ring stage parasites.

Fig. 4 – Indirect Immunofluoresence Assays (IFAs) of PfK2 and PfK2 in *P. falciparum*-infected Red Blood Cells. IFAs were performed on methanol/acetone fixed thin blood smears. The antibodies used were affinity purified anti-PfK1 F4 and anti-PfK2 F4, and anti-KAHRP and anti-MESA antisera. Representative IFAs of ring, trophozoite and schizont stage infected red blood cells are presented for each antibody. **A.** IFA of PfK1 shows no labeling in rings, and increasing red blood cell membrane surface labeling of trophoziote- and schizont-infected red blood cells. Predominantly

labeling appears to occur in a semi-punctate localization pattern. **B.** PfK2 was not detected in rings, with no to minimal detection in trophozoites. Schizonts showed intense foci of label, which appeared to correspond with the location of the developing merozoites within. **C.** KAHRP was detected in a classic punctuate pattern on the surface of trophozoite- and schizont-infected red blood cells. No labeling of KAHRP was detected in ring-infected red blood cells. D. More diffuse red cell membrane surface labeling of MESA was detected on trophozoite and schizont-infected red blood cells. No labeling was detected in ring-infected red blood cells.

Fig. 5 – Drug Assays. 72 hour [^{3}H] incorporation assays were performed to determine the parasites response to exposure to various K^+ channel blockers. Graphical plots of the mean ± standard error of the mean (SEM) for each drug dilution are shown. The IC_{50} and IC_{90} ± SEM for each drug were also determined (Table 3).

Table 1 – Oligonucleotide primer sequences. Sequences shown in upper case are gene specific. Underlined sequences indicate restriction sites. + and – indicate sense and antisense strand sequences, respectively.

Table 2 - Differential Parasitaemia counts for the Western Blot Parasite Samples. Differential parasitaemias were determined from Geimsa stained smears taken at the same time as the time point samples. Approximately 1000 total cells and the number of ring, trophozoite and schizont infected red blood cells were counted for each time point.

Table 3. Summary of IC_{50} and IC_{90} data. IC_{50} and IC_{90} drugs were obtained for various drugs using 72 hour [^{3}H] hypoxanthine incorporation assays. Three drugs, apamine (peptide found in bee venom and blocker of ATP-type Ca^{2+}-activated K^+ channels), charybdotoxin (recombinant peptide from scorpion venom (*Leiurus quinquestriatus*) and a potent inhibitor of Ca^{2+}-activated and voltage-gated K^+ channels) and verruclogen (isolated from *Penicillium verruculosum* and a blocker of high conductance Ca^{2+}-activated K^+ channels) were not pursued after initial assays, due to lack of detectable effect on [^{3}H] hypoxanthine incorporation (data not shown).

Table 1. Oligonucleotide primers.

Primer	Sequence (5' → 3')[a]	Target[b]
P1	cggga<u>attc</u>ATGAATAATGATAATATTGGGAG	PfK1 full length (+)
P2	cccaagcttTCAGACTTGGTCATGCGGTTC	PfK1 full length (-)
P3	ccggg<u>aattc</u>atTTTAATAATCATGATCATCAGAATC	PfK1 F3 (+)
P4	ccc<u>aagctt</u>TAAACATTCTTGATCATCTTTTTCC	PfK1 F3 (-)
P5	ccggg<u>aattc</u>tcAAAAAAGGACAAAACCATCAACTT	PfK1 F4 (+)
P6	ccc<u>aagctt</u>CATATTTTTTTCCATATCTTTAATG	PfK1 F4 (-)
P7	cgc<u>ggatcc</u>gATGAAAAGCGGATTATTTTCTATG	PfK2 full length (+)
P8	ccg<u>gaattc</u>TCACAATATATAAACTATATCATCGAATC	PfK2 full length (-)
P9	ccgg<u>gaattc</u>ATAATGATAATGATAATAATAATATTATA	PfK2 F3 (+)
P10	ccc<u>aagctt</u>tTTTAATTCCTTTTTATTATCAATTGT	PfK2 F3 (-)
P11	ccgg<u>gaattc</u>GAATATCAAGAATATTTACCAAAAAC	PfK2 F4 (+)
P12	ccc<u>aagctt</u>ATTATGAATATGAATATTTGTATTTGA	PfK2 F4 (-)

[a] Sequences shown in upper case are gene specific. Underlined sequences indicate restriction sites.
[b] + and – indicate sense and antisense strand sequences, respectively.

Table 2 - Differential Parasitaemia counts for the Western Blot Parasite Samples.

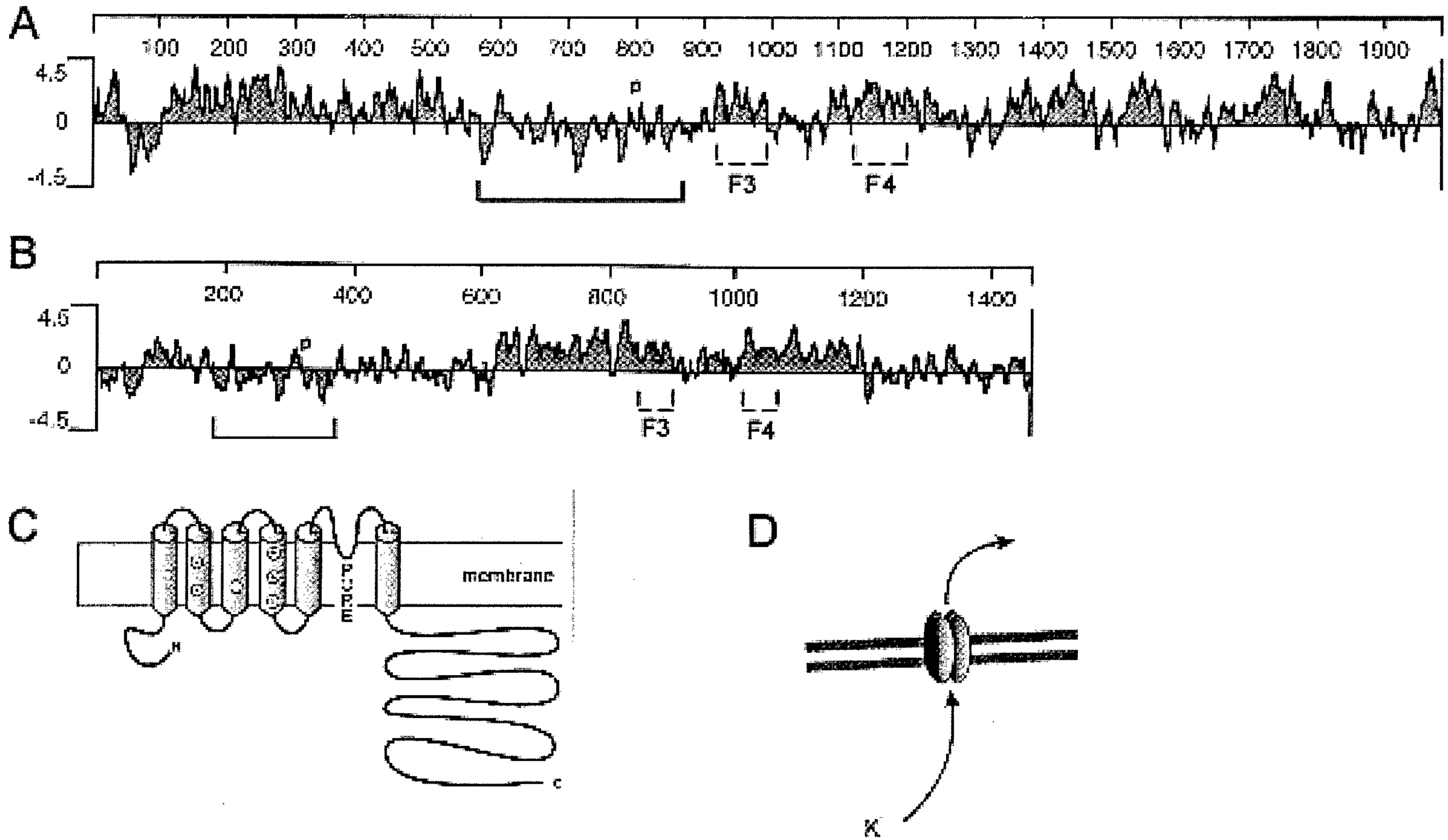
Sample	Total Parasitaemia[a]	Differential Parasitaemia[a]	Comments[b]
1	3.29%	86.7% Rings (R) 13.3% Trophozoites (T) 0.0% Schizonts (S)	Mid rings Early trophozoites
2	2.8%	15.6% R 81.3% T 3.1% S	Mid rings Early trophozoites
3	2.5%	3.3% R 96.7% T 0.0% S	Late trophozoites
4	3.1%	2.5% R 35.0% T 62.5% S	Late trophozoites Early/mid schizonts
5	5.8%	74.0% R 13.0% T 13.0% S	Late Schizonts
6	5.9%	90.2% R 5.6% T 4.2% S	Early trophozoites Late Schizonts
7	8.38%	74.5% R 25.5% T 0.0% S	Mid/late rings Early/mid trophozoites

[a] Differential parasitaemias were determined from Geimsa stained smears taken at the same time as the time point samples. Approximately 1000 total cells and the number of ring, trophozoite and schizont infected red blood cells were counted for each time point.

[b] Predominant parasites stage(s) present in samples.

Table 3. Summary of IC_{50} and IC_{90} data.

Drug	Activity	Clinical Use	IC_{50}	IC_{90}
Quinine	K^+ channel blocker	Anti-malarial	70.51 ± 4.3 nM	104.54 ± 3.5 nM
Quinidine	K^+ channel blocker	Anti-malarial, anti-arrhythmic drug	36.50 ± 1.3 nM	46.70 ± 1.0 nM
Bicuculline methiodide	Blocks Ca^{2+}-activated K^+ channels, $GABA_A$ receptor antagonist	**XXXX**	79.33 ± 2.9 μM	169.35 ± 7.9 μM
Clotrimazole	Blocks Ca^{2+}-activated K^+ channels	Anti-fungal drug	0.60 ± 0.01 μM	0.80 ± 0.01 μM
Haldol	Dopamine receptor antagonist	Anti-psychotic drug	4.29 ± 0.17 μM	10.58 ± 0.6 μM
Tubocurarine chloride	Nicotinic acetylcholine receptor antagonist	Non-depolarizing muscle relaxant, induces neuromuscular paralysis	129.01 ± 3.2 μM	187.86 ± 1.2 μM
Trifluoperazine hydrochloride	Calmodulin and dopamine receptor antagonist	Anti-anxiety, anti-psychotic drug	44.99 ± 1.0 μM	62.46 ± 2.3 μM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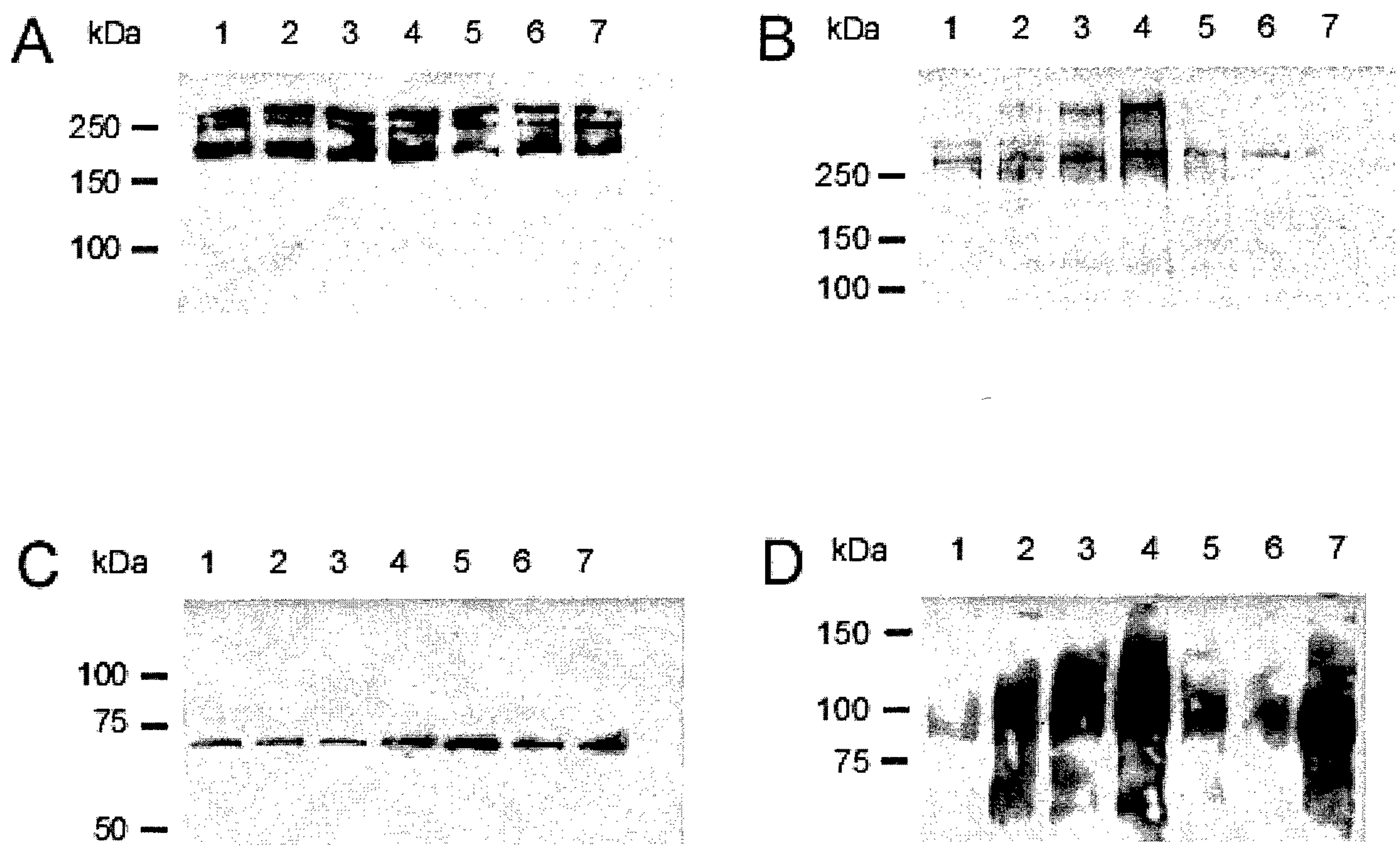
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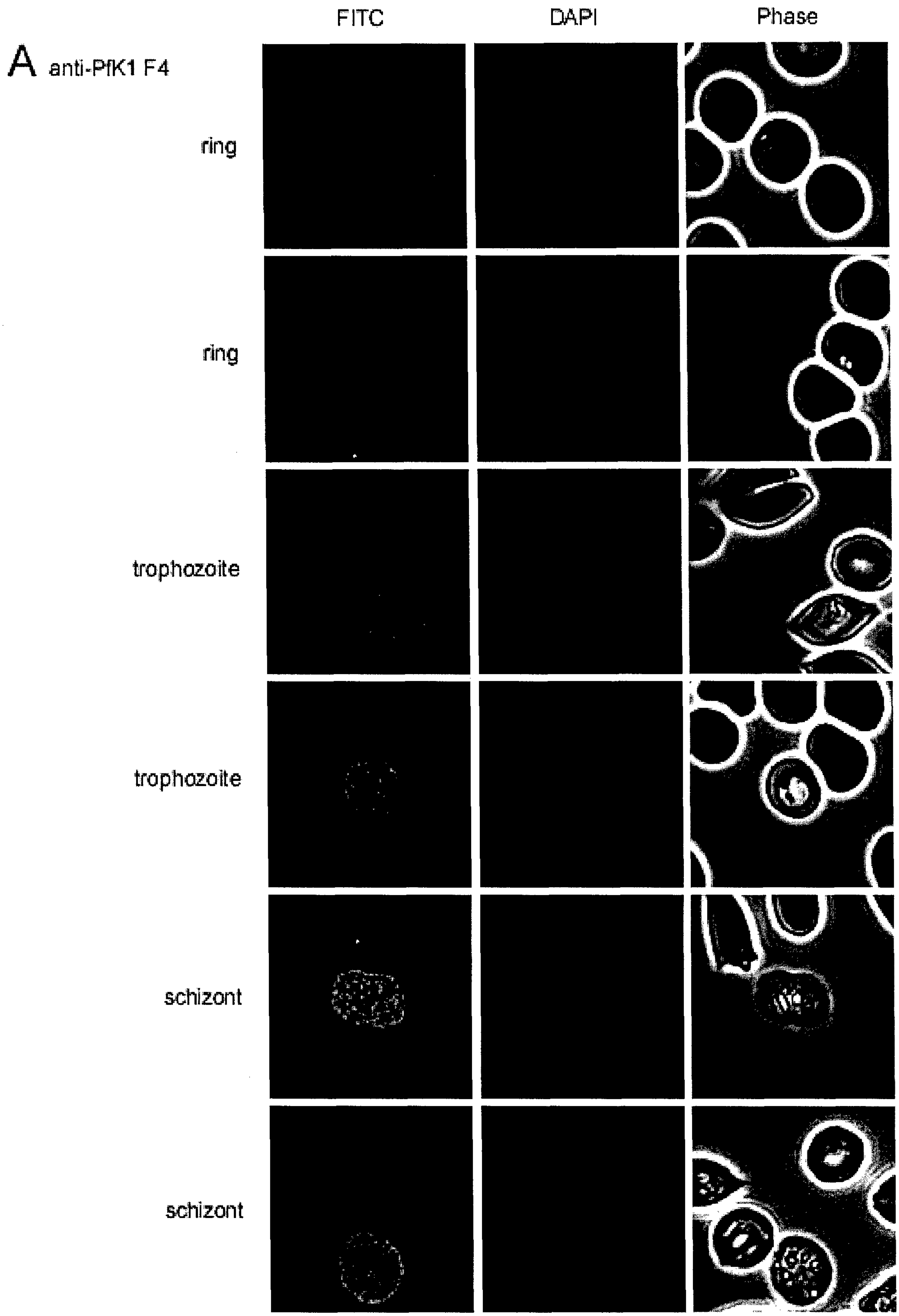
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DAPI
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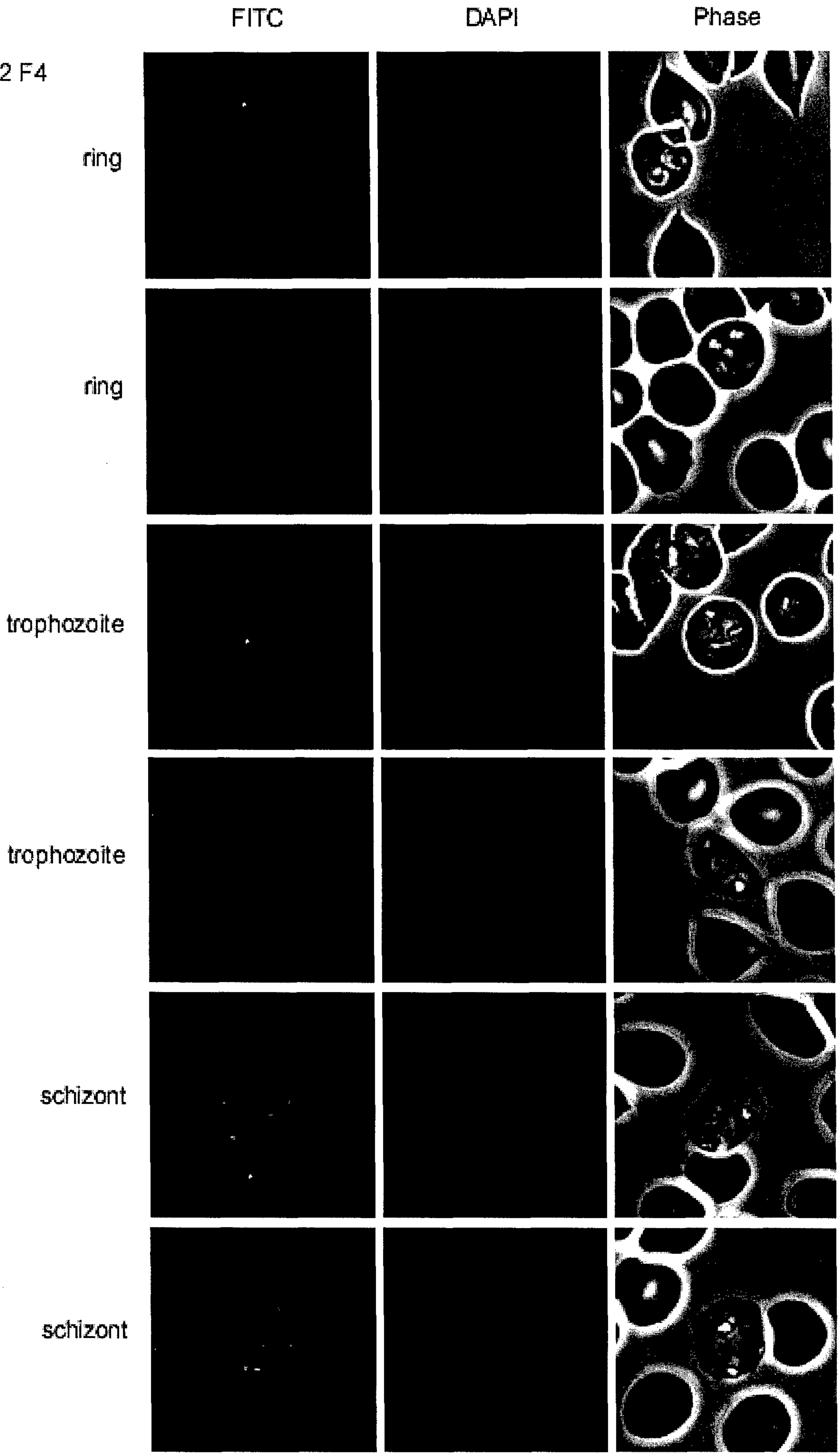
ring
ring
trophozoite
trophozoite
schizont
schizont

B anti-PfK2 F4



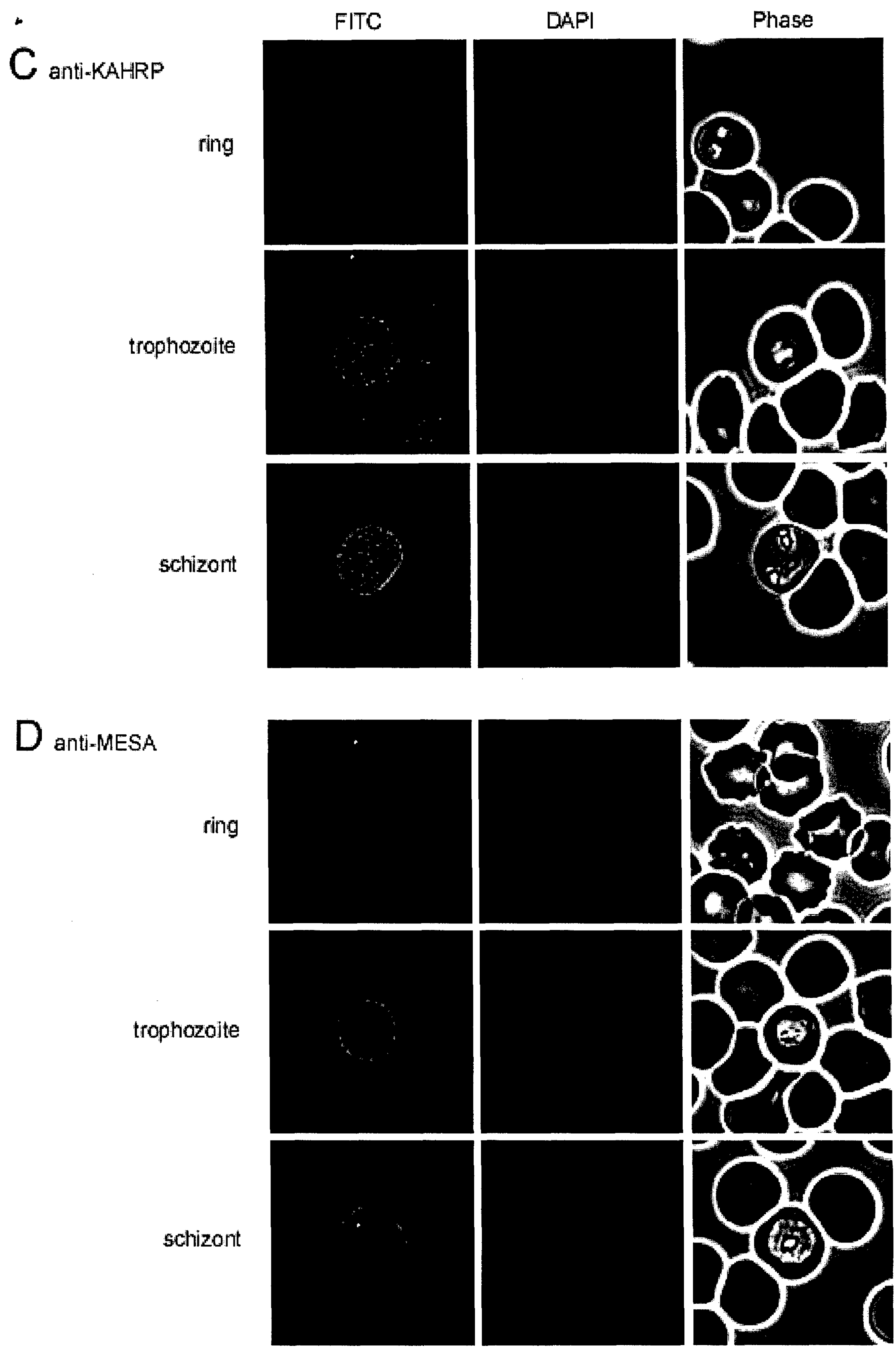

FITC
DAPI
Phase
C anti-KAHRP
ring
trophozoite
schizont
D anti-MESA
ring
trophozoite
schizont

Fig. 5 Growth Response Curves